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Highly sensitive liquid chromatography–electrospray mass spectrometry (LC–MS) method for the determination of etoposide levels in human serum and plasma

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Abstract

Etoposide is one of the most commonly used antineoplastic agents. A highly sensitive liquid chromatography–electrospray mass spectrometry (LC–MS) method was developed for the determination of etoposide in human serum and plasma. Etoposide was extracted with chloroform and extracts were reconstituted in acetonitrile followed by the evaporation of chloroform with nitrogen gas. Etoposide was separated using Lichospher 100 RP-18 (5 μm) column (250 mm×4 mm) with the mobile phase of acetonitrile–water containing 0.1% acetic acid (45/55, v/v) at flow-rate of 0.5 ml/min. Selected-ion monitoring (SIM) mode was performed on m/z 589 (positive ion mode) using a fragmentor of 75 V. Good linearity ($r>0.9965$) was observed between concentrations of 0.0125–5 μM in 200 μl serum and 0.01–10 μM in 100 μl plasma. Intra- and inter-assay variabilities were less than 7% and the lowest detection limit of etoposide was 0.005 μM in both serum and plasma at a signal-to-noise ratio of ~4. The etoposide concentrations in four cancer patients treated with etoposide are also presented to demonstrate the clinical utility of this new method, which should aid the pharmacokinetically guided use of etoposide in clinical settings.

Keywords: Etoposide

1. Introduction

Etoposide (VP-16) is a semi-synthetic derivative of podophyllotoxin (Fig. 1A). As a potent inhibitor of topoisomerase II, VP-16 is one of the most commonly used antineoplastic agents [1–4].

Currently, etoposide levels in biological fluids can be monitored using methods of high-performance liquid chromatography (HPLC)–UV [5,6], HPLC–fluorescence (F) detection [7–9] and HPLC–electrochemical detection (ECD) [10–13], or an ELISA method [14]. Etoposide levels can also be determined by LC–MS with ^{252}Cf fission fragment-induced ionization interface [15,16], however, this interface is not routinely available for MS spectrometry [17]. Here, we now report a highly sensitive quantitative detection method for VP-16 which employs HPLC–electrospray mass spectrometry using atomospheric pressure ionization (API) interface. The sensitivity and specificity of this novel LC–MS method for the quantitation of VP-16 surpasses all previous method using HPLC–UV, HPLC–F and

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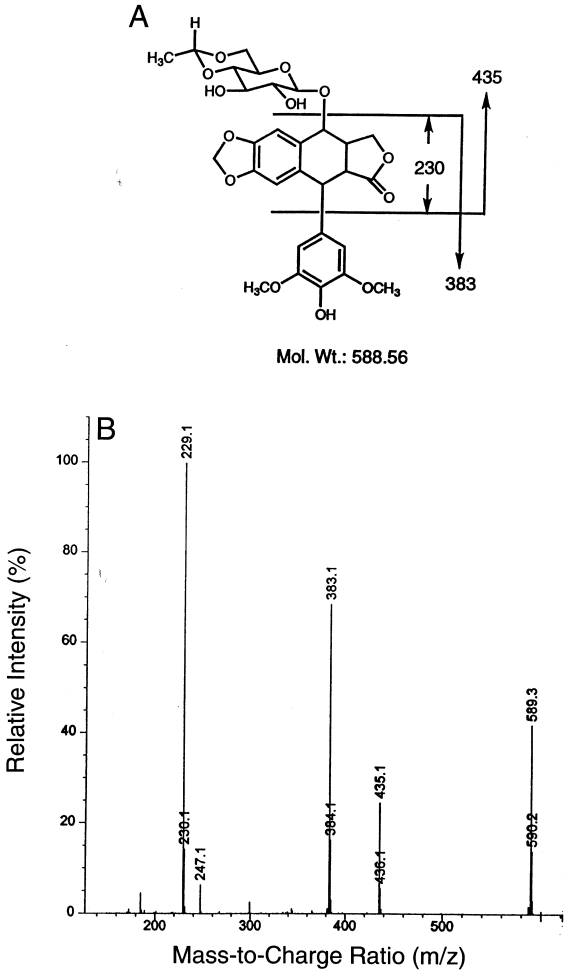

Fig. 1. (A) Chemical structures of etoposide. Molecular weight of etoposide was 588.5. (B) the mass spectrum of the etoposide molecular ion $[M+H]^+$ at m/z 589 and other fragment ions m/z at 229, 383, 435 and 247.

HPLC–ECD. Application of this method for measuring etoposide concentrations in four cancer patients demonstrates the applicability of the assay for clinical samples.

2. Materials and methods

2.1. Chemicals

All reagents used in this study were HPLC grade. Deionized distilled water was used thoughout the work (U.S. Filter, US Filter Corporation). Acetonitrile, chloroform, acetic acid were obtained from Fisher Chemicals (Fair Lawn, NJ, USA).

Etoposide was purchased from Sigma (St Louis, MO, USA). Stock solutions of etoposide were prepared in acetonitrile at a concentration of 1 m*M*, and stored at −20°C. The stock solutions were then diluted further to yield appropriate working solutions for generation of calibration curve.

2.2. Chromatographic conditions

The HPLC system (Hewlett-Packard, Palo Alto, CA, USA) consisted of a HP series 1100 instrument equipped with a quaternary pump, an autosampler, an automatic electronic degasser, an automatic thermostatic column compartment, a diode array detector and a computer with a Chemstation software program for data analysis [18–21].

A 250×4 mm Lichospher 100, RP-18 (5 μm) analytical column and a 4×4 mm Lichospher 100, RP-18 (5 μm) guard column were obtained from Hewlett-Packard. Acetonitrile–water containing 0.1% of acetic acid (45:55, v/v) was used as the mobile phase. The mobile phase was degassed automatically by the electronic degasser system. The column was equilibrated and eluted under isocratic conditions utilizing a flow-rate of 0.5 ml/min at ambient temperature. The wavelength of detection was set at 206 nm for etoposide. Peak width, response time and slit were set at >0.03 min, 0.5 s and 4 nm, respectively.

2.3. Mass spectrometric conditions

Mass spectrum analysis was carried out using atmospheric pressure ionization–electrospray (API–ES) and a high-energy-dynode (HED) electron multiplier (Hewlett-Packard, Palo Alto, CA, USA) [21]. High purity nitrogen gas was provided by Nitrogen Generator (Hewlett Pachard). The conditions for mass spectrum analysis were set at a fragmentor voltage of 75 V, drying gas flow of 10 l/min, nebulizer pressure of 25 p.s.i.g and drying gas temperature of 350°C. Selected-ion monitoring (SIM) mode was performed on m/z 589 (positive ion mode) using a dwell time of 586 ms with the capillary voltage at 3500 V.

2.4. Extraction procedures

For extraction of etoposide levels from 200 μl serum samples or 100 μl plasma samples, 7 ml of chloroform was added to the serum sample, and the mixture was vortexed thoroughly for 3 min. Following centrifugation (300 *g*, 5 min), the aqueous layer was frozen using acetone–dry ice and the organic phase was transferred into a clean test tube. The chloroform extracts were dried under a slow steady stream of nitrogen gas. The residue was reconstituted in 50 μl of acetonitrile and a 10 μl aliquot of this solution was injected for HPLC analysis. All extraction procedures were performed at room temperature.

2.5. Extraction recovery

Replicate ($N=5$) serum samples (200 μl per sample) were spiked with known amounts of etoposide to yield final etoposide concentrations of 0.0375, 0.375 and 3.75 μ*M* in serum and of 0.075, 0.75 and 7.5 μ*M* in plasma. Using the extraction procedures and analytical methods described above, the extraction recovery (ER) was calculated according to the formula:

$$\%\,\mathrm{ER} = \frac{\text{Peak Area[etoposide]}_{\text{extracted}}}{\text{Peak Area[etoposide]}_{\text{unextracted}}} \times 100$$

2.6. A calibration curve

A calibration curve was generated to confirm the linear relationship between the peak area of etoposide, and the concentration of etoposide in the test samples. Etoposide was added to serum to yield final concentrations of 0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, and 5.0 μ*M* and to plasma to yield final concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10 μ*M*, subsequently, the serum or plasma samples with known amounts of etoposide were extracted as described above, and the standard curves were generated by plotting the peak area of etoposide in the mass spectrum detector against the drug concentrations tested. Unweighted linear regression analysis of the standard curve was performed by using the CA-Cricket Graph III computer program, Version 1.1 (Computer Association, Inc., Islandia, NY, USA). The linearity was confirmed using the Instat Program V3.0 (GraphPad Software, San Diego, CA, USA).

2.7. Intra-assay and inter-assay accuracy and precision

To evaluate the intra-assay accuracy and precision, etoposide was added to drug-free serum at concentrations of 0.375 and 3.75 μ*M*, and to drug-free plasma at concentrations of 0.075, 0.075 and 7.5 μ*M*. These standard samples were prepared and analyzed within 24 h for calculating intra-assay accuracy and precision. The concentrations were calculated using a standard curve. The ratio of the calculated concentration over the known concentration of etoposide was used to determine the accuracy of the analytical method, and the coefficient of variance was used as an index of precision. The inter-assay accuracy and precision were determined in four independent experiments. One-way analysis of variance (ANOVA) was performed using the Instat Program V3.0 to determine the statistical significance of differences between expected vs. measured etoposide levels in spiked samples.

2.8. Patient samples and pharmacokinetic analysis

Blood samples were obtained from four cancer patients, who received etoposide intravenously via 1-h infusion at dose of 50 $\mathrm{mg/m^2}$. Informed consent for chemotherapy was obtained from all patients or their guardians according to Department of Health and Human Services Guidelines.

Pharmacokinetic modeling and pharmacokinetic parameter estimations were carried out using the pharmacokinetics software, WinNonlin Program, Professional version 3.0 (Pharsight, Mountain View, CA, USA) [18,21]. An appropriate pharmacokinetic model was chosen on the basis of lowest sum of weighted squared residuals, lowest Schwartz Criterion (SC), lowest Akaike's Information Criterion (AIC) value, lowest standard errors of the fitted parameters, and dispersion of the residuals. The elimination half-life was estimated by linear regression analysis of the terminal phase of the concentration–time profile. The area under the concentration time curve (AUC) was calculated by the

linear trapezoidal rule between first (0 h) and last sampling time plus C/k, where C is the concentration of last sampling and k is the elimination rate constant. The systemic clearance (Cl) was determined by dividing the dose by the AUC. The mean residence time (MRT) was calculated by dividing the area under the first moment curve (AUMC) by AUC.

3. Results and discussion

3.1. Mass spectrum for etoposide

The mass spectrum for etoposide was determined with the scanning mode, and we found that an m/z value of 589 $[M+H]^+$ was associated with the parent compound etoposide (Fig. 1B). Other fragment ions such as m/z of 229 and m/z of 383 had a higher abundance (fragment patterns shown in Fig. 1A). These fragment ions may also be produced from metabolites such as etoposide catechol [11,22]. Therefore, we set the m/z at 589 in SI*M* mode for detecting parent etoposide in biological samples. With this SIM mode, the detection of etoposide is extremely specific, since only the etoposide molecular ion is detected at m/z 589. In comparison to HPLC–UV, HPLC–F and HPLC–ECD, LC–MS is more specific in detecting the etoposide molecule. Although HPLC–ECD is the most commonly used method, the electrode is easily contaminated. We have difficulties to obtain reproducible results by using HPLC–ECD to quantitate etoposide levels in plasma (data not shown).

3.2. Selection of fragmentor voltage and flow rate of mobile phase

We next examined the effect of the fragmentor voltage on the absolute peak area of etoposide. To this end, the fragmentor voltage was varied between 25 V and 125 V. The highest peak area for etoposide was obtained at a fragmentor voltage of 75 V (Fig. 2A). Voltages higher than 75 V resulted in a lower peak area at m/z 589, likely due to fragmentation of etoposide.

We also examined the effect of the flow-rate of the mobile phase on the sensitivity and speed of the detection method (Fig. 2B). Flow rates higher than

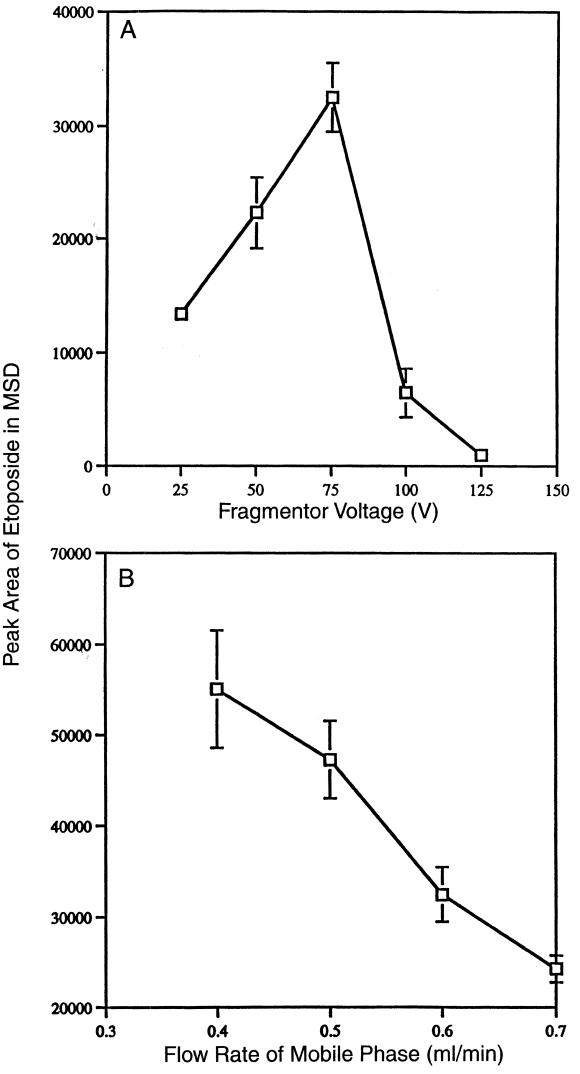

Fig. 2. Effects of fragmentor voltage (A) and flow-rate (B) on the sensitivity of the etoposide detection method.

0.5 ml/min decreased the sensitivity. Flow rates lower than 0.5 ml/min were accompanied by longer HPLC separation times. In consideration of both sensitivity and separation time, we chose a flow-rate of 0.5 ml/min for the mobile phase.

3.3. Sensitivity and accuracy of the HPLC detection method for etoposide levels

Under the described chromatographic conditions, the retention time for etoposide was 6.1 min. At the

retention time, etoposide was eluted without an interference peak from the blank serum (Fig. 3B2 and 3A2). The MS detection method was more specific than the UV detection method (Fig. 3B1 vs. 3B2). With the described extraction conditions, the recovery (mean±SD, $N=5$) of etoposide from 200 μl serum samples at 0.0375 μ*M*, 0.375 μ*M* and 3.75 μ*M* was 82.5±4.4%, 83.1±2.8% and 88.6±1.9%. The average extraction recovery from 200 μl serum was 84.9±4.2%. With the same extraction conditions, the recovery (mean±SD, $N=5$) of etoposide from 100 μl plasma samples at 0.075 μ*M*, 0.75 μ*M* and 7.5 μ*M* was 87.7±8.3%, 86.2±5.8% and 93.4±8.1%, with an average extraction recovery of 89.1±8.1%.

The standard curves obtained from extraction of serum samples containing known amounts of etoposide were linear ($r>0.9965$) over the concentration ranges tested (i.e. 0.0125–0.5 μ*M* and 0.5 –5 μ*M* in serum and also linear over the concentration ranges tested (i.e. 0.01–0.5 μ*M* and 0.5–10 μ*M* in plasma (Table 1). The linearity was statistically confirmed using the Instat Program V3.0. At a signal-to-noise ratio of ~4, the lowest limits of detection of etoposide were 0.005 μ*M* in both serum and plasma samples. The therapeutic plasma concentration of

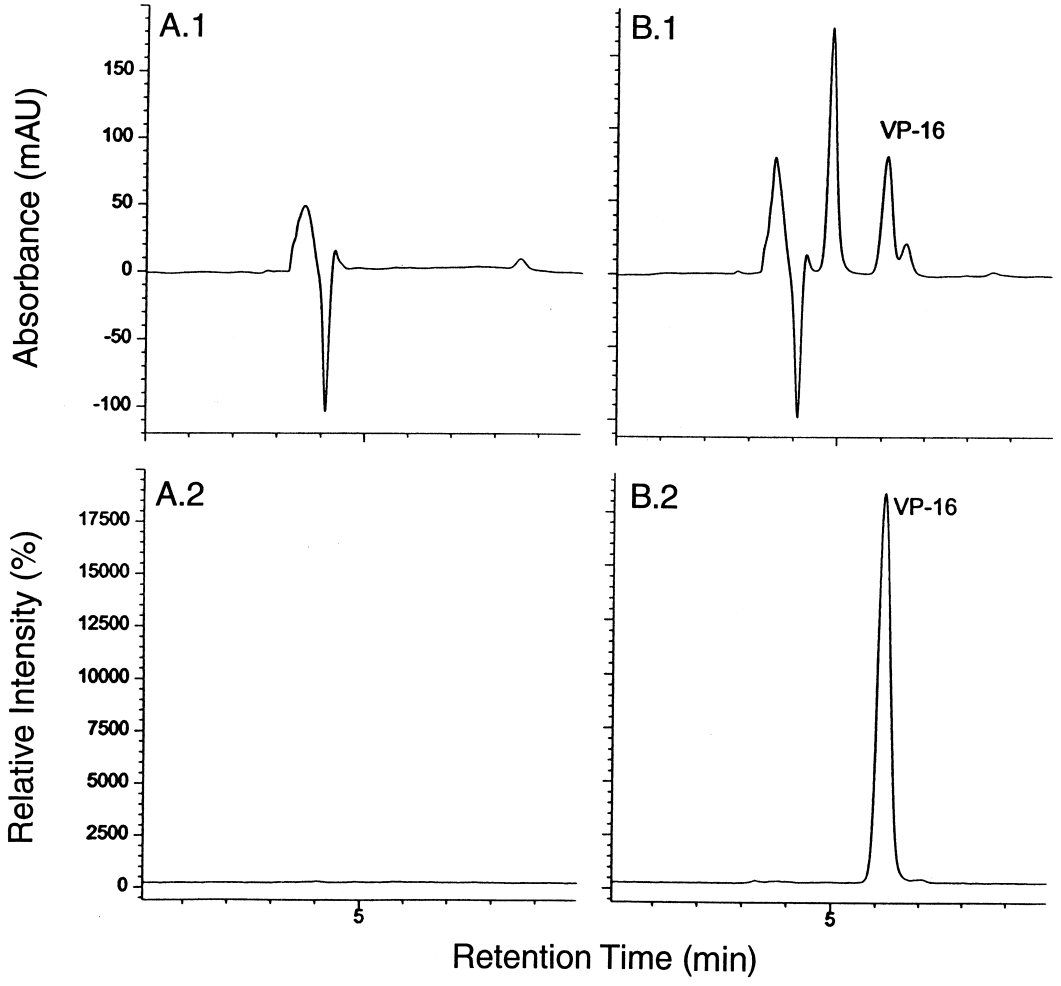

Fig. 3. Representative chromatograms from blank serum [A1 (UV) and A2 (MSD)] and from serum sample of a patient treated with etoposide [B1 (UV) and B2 (MSD)].

Table 1
Least squares regression analysis of etoposide standard curve in serum and plasma[a]

Concentration ranges	Slope (μM/Abundance)	Y-intercept (μM)	Correlation coefficient
Serum ($N=4$)			
0.0125–0.5 μM	$8.1\times10^{-6}\pm1.31\times10^{-6}$	$1.9\times10^{-3}\pm5.1\times10^{-4}$	0.9988±0.0001
0.5–5 μM	$10.3\times10^{-6}\pm9.5\times10^{-7}$	0.238±0.138	0.9970±0.0018
Plasma ($N=6$)			
0.01–0.5 μM	$1.4\times10^{-5}\pm6.9\times10^{-6}$	$1.4\times10^{-3}\pm5.6\times10^{-3}$	0.9972±0.0034
0.5–10 μM	$1.9\times10^{-5}\pm8.0\times10^{-6}$	−0.138±0.214	0.9965±0.0035

[a] Data are presented as mean±SD.

etoposide is approximately 1 μM (between 0.8–1.7 μM) [23], the described LC–MS method is sensitive enough to detect effective pharmacological etoposide concentrations in serum or plasma.

The intra- and inter-assay coefficients of variance (C.V.) were less than 7%. The average accuracy of this detection method was 99.7% in serum and 98.3% in plasma (Table 2). There was no statistically significant difference by ANOVA analysis between expected and experimentally determined etoposide levels in spiked serum or plasma samples.

3.4. Etoposide pharmacokinetics in patients treated with etoposide

We next used the described LC–MS method to determine the serum and plasma etoposide levels in four cancer patients who received 50 mg/m^2 etoposide intravenously over a 1-h infusion. The unknown samples were processed and analyzed in parallel with the spiked serum or plasma calibrators (the concentrations for the standard curve ranged from 0.01 to 10 μM). The representative etoposide concentrations–time curve presented in Fig. 4 shows that etoposide concentration versus time changes can be best described using a single compartment pharmacokinetic model. The pharmacokinetic parameter values are shown in Table 3. The predicted maximum serum etoposide concentration (C_{max}) and AUC [mean±SD (ranges)] were 12.1±3.5 μM (7.9–17.4 μM) and 3710.8±707.8 μM/min (2506.7–4306.4 μM/min). The elimination half-life and mean residence time (MRT) were 199.3±36.4 min (149.9–252.7 min) and 287.6±52.5 min (216.4–

Table 2
Intra-assay and inter-assay accuracy and precision of etoposide in serum[a]

	Added	Found	Accuracy (%)	C.V. (%)[b]
Serum				
Intra-assay ($n=5$)				
	0.375 μM	0.368±0.013	98.0±3.5	3.6
	3.75 μM	3.905±0.044	104.1±1.2	1.1
Inter-assay ($n=4$)				
	0.375 μM	0.360±0.010	96.0±2.8	2.9
	3.75 μM	3.781±0.078	100.8±2.1	2.0
Plasma				
Intra-assay ($n=4$)				
	0.075 μM	0.071±0.004	95.2±5.7	6.0
	0.75 μM	0.743±0.05	99.0±6.7	6.8
	7.5 μM	7.15±0.32	95.3±4.3	4.5
Inter-assay ($n=4$)				
	0.075 μM	0.0743±0.004	99.1±5.6	5.7
	0.75 μM	0.746±0.015	99.4±2.0	2.0
	7.5 μM	7.623±0.417	101.6±5.6	5.5

[a] Data are expressed as mean±SD.
[b] C.V.=coefficient of variance.

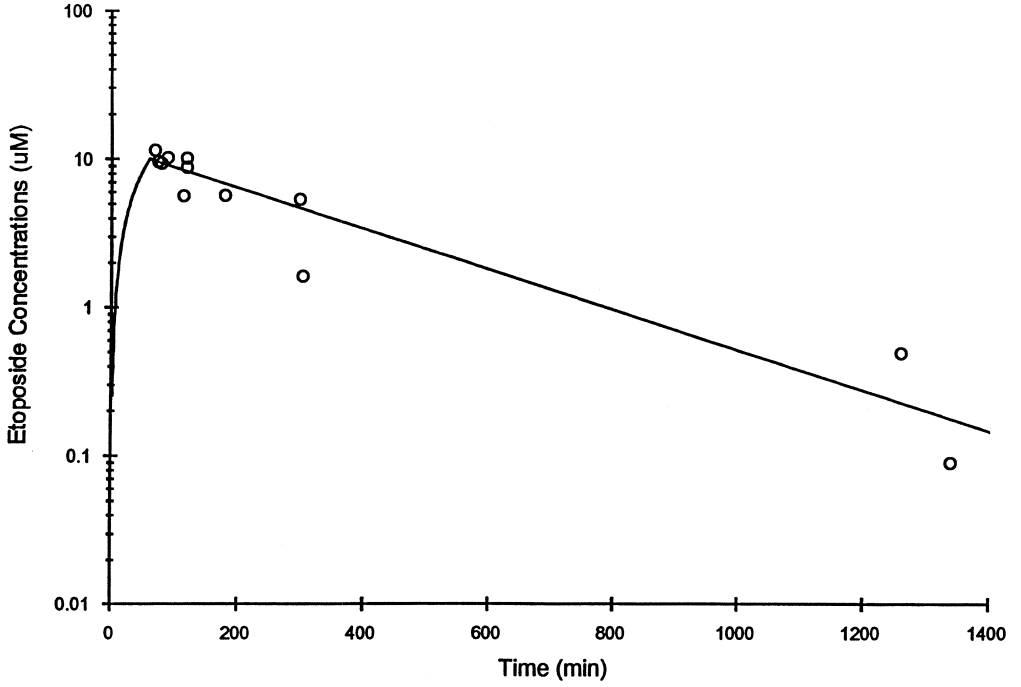

Fig. 4. Composite plasma etoposide concentration–time profile in three representative patients treated with 50 mg/m^2 etoposide intravenously via a 1-h infusion.

364.6 min). The systemic clearance was 24.0±5.8 ml/min/m^2 (19.7–33.9 ml/min/m^2).

In summary, we have developed a highly sensitive and accurate analytical HPLC method for the quantitative detection of etoposide in clinical serum and plasma samples. The sensitivity of LC–MS for the quantitation of VP-16 surpasses that of any HPLC–UV, HPLC–fluorescence and HPLC–electrochemical detection method. Moreover, we have successfully applied the LC–MS method to determine the serum and plasma etoposide levels in cancer patients treated with etoposide. The availability of this new assay should aid the pharmacokinetically guided use of etoposide in clinical settings.

Table 3
Estimated pharmacokinetic parameter values of etoposide in cancer patients

PK Parameters[b]	Clinical Samples				All Samples (Mean±SD)
	PT 1[a] (Serum)	PT 2 (Plasma)	PT 3 (Plasma)	PT 4 (Plasma)	
Dose (mg/m^2)	50	50	50	50	
AUC (μ*M**min)	4306.4	4098.9	2506.7	3931.1	3710.8±707.8
$t_{1/2}$ (min)	149.9	252.7	198.1	196.4	199.3±36.4
C_{max} (μ*M*)	17.4	10.4	7.9	12.5	12.1±3.5
Cl (ml/min/m^2)	19.7	20.7	33.9	21.6	24.0±5.8
MRT (min)	216.4	364.6	285.8	283.4	287.6±52.5
V_{ss} (ml/m^2)	4270.6	7560.5	9691.4	6127.7	6912.6±1983.6

[a] PT = patient.
[b] PK = pharmacokinetic.

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